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(71) Applicant: UNIVERSITY OF SOUTH FLORIDA [US/US]; 4202 Fowler Ave., Tampa, FL 33620-6250 (US).

(71)(72) Applicant and Inventor: GRASSO, Robert, J. [US/US]; 15816 Deep Creek Lane, Tampa, FL 33624 (US).

(74) Agents: MASON, Joseph, C., Jr. et al.; 1307 U.S. 19 South, Suite 102, Clearwater, FL 34624 (US).

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(54) Title: METHOD FOR ELECTROFUSING BIOLOGICAL PARTICLES TO TISSUES

(57) Abstract

This invention relates to methods for electrofusing biological particles to cells of animal or plant tissues *in vitro*, *in situ*, or *vivo*. Selected biological particles are placed in contact with cells of a selected tissue and fused to cells in the tissue by means of an electric field. The method of this invention may be used to produce animals and plants characterized by features and properties that differ from those typically displayed by the native species. One embodiment of the methods of this invention has been used to produce an animal model for ocular gonorrhea by electrofusing human cells with functional receptors for the human bacterial pathogen *Neisseria Gonorrhoeae* to epithelial cells in the histologically intact superficial corneal tissue of living rabbits. The methods of this invention may be used to produce other animal or plant models for the study of receptor-mediated processes, including infectivity studies required for the development of vaccines effective to protect against or lessen the severity of infections caused by microbial pathogens. This is achieved by electrofusing cells with membrane receptors for a pathogen to cells of tissues of plants or animals that are normally not susceptible to infection by that pathogen. Other applications are disclosed.

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Method for Electrofusing Biological Particles to Tissues

Technical Field

This invention relates to methods for electrofusing biological particles to cells of animal or plant tissues *in vitro*, *in situ*, or *in vivo*. Selected biological particles are placed in contact with cells of a selected tissue and fused to cells in the tissue by means of an electric field. The methods of this invention may be used to produce animals and plants characterized by features and properties that differ from those typically displayed by the native species. One embodiment of the methods of this invention has been used to produce an animal mode for ocular gonorrhea by electrofusing human cells with functional receptors for the human bacterial pathogen *Neisseria gonorrhoeae* to epithelial cells in the histologically intact superficial corneal tissue of living rabbits. The methods of this invention may be used to produce other animal or plant models for the study of receptor-mediated processes, including infectivity studies required for the development of vaccines effective to protect against or lessen the severity of infections caused by microbial pathogens. This is achieved by electrofusing cells with membrane receptors for a pathogen to cells of tissues of plants or animals that are normally not susceptible to infection by that pathogen. Other applications are disclosed.

Background Art

Various methods and apparatus for fusing cells and other biological particles in vitro are known in the art. Conventional fusion techniques employ chemical or biological agents known as fusogens that induce discrete cells to fuse and form single hybrid cells that contain the genetic information of both the original cells within a single cell membrane that includes components of the membranes of the original cells. Polyethylene glycol is a well-known chemical fusogen that has been employed to create a variety of fusion products, including hybridomas for the production of monoclonal antibodies. Viral fusogens, such as Sendai viruses, have also been used to create fusion products such as hybridomas. Some of the literature relating to the use of chemical and viral fusogens for the production of hybridomas is reviewed by Westerwoudt, R. J. in "Improved Fusion Methods, IV. Technical Aspects," J. Immunol. Meth., 77, pp. 181-96 (1985).

Although conventional chemical and viral fusion techniques are useful, their utility is limited because the fusogens often cause extensive damage that affects cell viability. The processes are also difficult to control and are characterized by low yields of fusion products. In addition to control and cytotoxicity problems, chemical fusogens cause cells to agglutinate nonspecifically. Although cells must be brought into contact for fusion to occur, nonspecific agglutination limits the possibility of

obtaining specific, desired fusion products. These and other limitations inherent in conventional chemical and viral fusion techniques are outlined by Zimmermann, U. and Vienken, J. in "Electric Field-Induced Cell-to-Cell Fusion," J. Membrane Biol., 67, pp. 165-82 (1982), and by Bates, G. W., Saunders, J. A. and Sowers, A. E. in Cell Fusion (Ed., Arthur E. Sowers), pp. 367-95 (Plenum Press, N.Y. 1987).

Recently, newly-developed electrofusion techniques have also been employed to induce fusion of cells and other biological particles in vitro. These electrofusion methods, which overcome many of the limitations of classical chemical and viral fusion techniques, generally include the following steps. Cells to be fused are placed in suspension in a non-electrolyte medium. The cell suspension is placed in a specially-constructed fusion chamber that includes electrodes connected to a source of electric power. The cell suspension is subjected to a non-uniform alternating current field created by applying alternating current to the electrodes in the fusion chamber. The non-uniform electric field induces the cells in suspension to act as dipoles. By the process of dielectrophoresis, the "dipole-cells" align themselves in the inhomogeneous electric field and move toward regions of higher field strength. As the cells align and migrate toward regions of higher field strength, they are attracted to each other and form strings of contiguous cells that are usually described as "pearl chains." After the cells have aligned and formed pearl chains, one or more short pulses of direct

obtaining specific, desired fusion products. These and other limitations inherent in conventional chemical and viral fusion techniques are outlined by Zimmermann, U. and Vienken, J. in "Electric Field-Induced Cell-to-Cell Fusion," J. Membrane Biol., 67, pp. 165-82 (1982), and by Bates, G. W., Saunders, J. A. and Sowers, A. E. in Cell Fusion (Ed., Arthur E. Sowers), pp. 367-95 (Plenum Press, N.Y. 1987).

Recently, newly-developed electrofusion techniques have also been employed to induce fusion of cells and other biological particles in vitro. These electrofusion methods, which overcome many of the limitations of classical chemical and viral fusion techniques, generally include the following steps. Cells to be fused are placed in suspension in a non-electrolyte medium. The cell suspension is placed in a specially-constructed fusion chamber that includes electrodes connected to a source of electric power. The cell suspension is subjected to a non-uniform alternating current field created by applying alternating current to the electrodes in the fusion chamber. The non-uniform electric field induces the cells in suspension to act as dipoles. By the process of dielectrophoresis, the "dipole-cells" align themselves in the inhomogeneous electric field and move toward regions of higher field strength. As the cells align and migrate toward regions of higher field strength, they are attracted to each other and form strings of contiguous cells that are usually described as "pearl chains." After the cells have aligned and formed pearl chains, one or more short pulses of direct

current are applied to the electrodes to cause electrical breakdown of the cell membranes of contiguous cells. After direct current fusion pulses have been applied, the non-uniform alternating current field is restored to keep the cells aligned and in contact while the cell membranes of contiguous cells coalesce and fuse to form single hybrid cells. These electrofusion methods have been used to create a variety of somatic cells hybrids in vitro. Common applications include the creation of hybridomas for the production of monoclonal antibodies (Vienken, J. and Zimmermann, U., FEBS Lett., 182, p. 278-80 (1985); Karsten, U., et al., Cancer Clin. Oncol., 310, pp. 733-40 (1985); Lo, M.M.S., et al., Nature, 310, pp. 792-94 (1984)) and the creation of other hybrids for genetic studies (Podesta, E. J., et al., Eur J. Biochem., 145, pp. 329-32 (1984); Finaz, C., et al., Exp. Cells Res., 150, pp. 477-82 (1984)). The same general technique has also been employed to fuse large liposomes. Buschl, R., et al., FEBS Lett., 150, pp. 38-42 (1982).

Cell fusion chambers and other apparatus used to practice such electrofusion methods have been described in the literature. E.g., United States Patent No. 4,476,004; United States Patent No. 4,578,167; United States Patent No. 4,578,168; United States Patent No. 4,561,961; United States Patent No. 4,622,302; Bates, G. W., et al., Cell Fusion (Ed., Arthur E. Sowers), pp. 367-95 (Plenum Press, N.Y. 1987); Zimmermann, U. and Vienken, J., "Electric Field-Induced

Cell-to-Cell Fusion, "J. Membrane Biol., 67, pp. 165-82 (1982); Zimmermann, U., "Electrical Breakdown, Electropermeabilization and Electrofusion," Rev. Physiol. Biochem. Pharmacol., 105, pp. 175-256 (1986).

In vitro cell electrofusion processes employing inhomogeneous alternative current fields to align the cells and cause formation of pearl chains of contiguous cells prior to application of fusion pulses have to some extent overcome the control and cytotoxicity problems of classical chemical and viral fusion techniques. These electrofusion techniques, however, are themselves subject to a number of problems and limitations, most of which arise out of the use of a non-uniform alternating current field. For example, Joule heating of the cell suspension, a result of current flow in the medium caused by application of the alternating current field, causes general damage to the cells as well as turbulence in the suspension that interferes with and reduces the stability of the cell-to-cell contact required for fusion. Although Joule heating may be reduced by using nonionic media with low conductivity, many cells are unable to tolerate such media for any useful length of time. The alternating current field also induces the cells in suspension to rotate or spin. Cell rotation interferes with membrane contact in the "pearl chains" and may adversely affect the fusion process. Another limitation of the method arises because the dielectrophoretic force required to align cells and form "pearl chains" depends on the size of the

particle. Smaller particles require stronger fields to align the particles and cause formation of pearl chains. Such high alternating current voltages are required for particles with a radius smaller than 0.5 μm that the process becomes impractical, particularly for the fusion of small liposomes.

Use of the dielectrophoresis cell-to-cell electrofusion method has been limited to in vitro fusion of suspended cells or other biological particles in specially-built microscope slides or fusion chambers. The size of available fusion chambers limits the number of cells that may be fused and the number of fusion products that may be recovered.

The theory, prinicples, applications, problems and limitations of state of the art in vitro cell-to-cell electrofusion techniques have been extensively reviewed. E.g., Bates, G. W., et al., Cell Fusion (Ed., Arthur E. Sowers), pp. 367-95 (Plenum Press, N.Y. 1987); Zimmermann, U., "Electrical Breakdown, Electropermeabilization and Electrofusion," Rev. Physiol. Biochem. Pharmacol., 105, pp. 175-256 (1986); Zimmermann, U., et al., "Electrofusion of Cells," Investigative Microtechniques in Medicine and Biology (Eds., J. J. Chayen and L. Bitensky), pp. 89-167 (Marcel Dekker, N.Y. 1984); Arnold W. M. and Zimmermann, U., "Electric Field-Induced Fusion and Rotation of Cells," Biological Membranes, (Ed., D. Chapman), Vol. V, pp. 389-454 (Academic Press, London 1984); Zimmermann, U., "Electric Field-Mediated Fusion and Related Electrical Phenomena," Biochim. Biophys. Acta, 694, pp. 227-77 (1982); Zimmermann,

U. and Vienken, J., "Electric Field-Induced Cell-to-Cell Fusion," J. Membrane Biol., 67, pp. 165082 (1982); Zimmermann, U., et al., "Cells with Manipulated Functions: New Perspectives for Cell Biology, Medicine, and Technology," Angew. Chem. Int. Ed. Engl., 20, pp. 325-44 (1981).

Although a number of alternative means for bringing about the cell juxtaposition and membrane contact required for electrofusion have been explored, the use of an inhomogeneous alternating current field so far has proven to be the most efficient and practical approach for general application in vitro. In 1978, Zimmermann and Pilwat reported that erythrocytes may be electrofused by subjecting a dense suspension of erythrocytes to electric fusion pulses. Zimmermann, U. and Pilwat, G., Sixth International Biophysics Congress, Kyoto 1978, Abstr. IV-19-(H), p 140. Use of a dense cell suspension apparently enhanced the chance that cells in the suspension would be in contact with one another, but the yield of fusion products was extremely low.

In 1979, Senda reported the fusion of two protoplasts that were pushed together by micropipettes and subjected to a fusion pulse. Senda, M., et al., Plant Cell Physiol., 20, pp. 1441-43 (1979). The fused cell was not viable and the procedure is plainly not practical for the fusion of large numbers of cells.

Other investigators have used chemical or viral agents to cause cells to adhere to one another, and subsequently fused

the juxtaposed cells by applying electric fusion pulses. E.g., Neumann, E., et al., Naturwissenschaften, 67, pp. 414-15 (1980); Weber, H., et al., Current Genetics, 4, pp. 165-66 (1981). This approach, however, is subject to some of the same limitations discussed above in connection with classical chemical and viral fusion techniques, including the problem of uncontrolled nonspecific agglutination.

Electrofusion of 3T3 cells (Teissie, J., et al., "Electric Pulse-Induced Fusion of 3T3 Cells in Monolayer Culture," Science, 216, pp. 537-38 (1982)) and electrofusion of CHO cells (Blangero, C. and Teissie, J., "Homokaryon Production by Electrofusion: A Convenient Way to Produce a Large Number of Viable Mammalian Fused Cells," Biochem. Biophys. Res. Comm., 114(2), pp. 663-69 (1983); Orgambide, G. et al., "Electrofusion of Chinese Hamster Ovary Cells After Ethanol Incubation," Biochim. Biophys. Acta, 820, pp. 58-62 (1985)) has been accomplished by subjecting monolayer cultures of those cells to fusion pulses after the requisite cell juxtaposition and membrane contact had been obtained by allowing the cultures to grow to confluence. This approach is characterized by disadvantages that make it difficult to efficiently produce specific, desired fusion products. First, there is no assurance that any significant number of cells or any particular cell in the culture will grow close enough to other cells to achieve the membrane contact required for electrofusion. Second, this approach does not provide the investigator with any control over which of the

cells in the monolayer will be electrofused. All cells in the monolayer are subjected to the fusion pulses that are applied. Finally, because cells in the monolayer may be in contact with more than one other cell, fusion pulses often produce hybrid "giant cells" that contain three or more nuclei within a single membrane.

The cell membrane contact required for electrofusion has also been obtained with two different centrifugation techniques. In the first approach, fusion was achieved by applying fusion pulses to a cell pellet obtained by centrifugation. Zimmermann, U., "Electric Field-Mediated Fusion and Related Electrical Phenomena," Biochim. Biophys. Acta, 694, pp. 227-77 (1982). In the second approach, low density cell suspensions were subjected to fusion pulses before centrifugation. Fusion of the cells occurred in the pellet formed by centrifuging cells that had already been rendered "fusogenic" by exposure to the fusion pulses. E.g., Zimmermann, U., et al., "Electrofusion: A Novel Hybridization Technique," Advances in Biotechnological Processes (Eds., A. Mizrahi, A. L. Van Wezel), 4, pp. 79-150 (Alan Liss, N.Y. 1985); Teissie, J. and Rols, M. P., "Fusion of Mammalian Cells in Culture Is Obtained by Creating the Contact Between Cells after Their Electropermeabilization," Biochem. Biophys. Res. Comm., 140(1), pp. 258-66 (1986). Because fusion occurs in the pellet in both of these centrifugation techniques, the investigator cannot monitor the process by optical means. These centrifugation techniques are also

marked by the same control and efficiency problems discussed above in connection with the description of the confluent monolayer culture approach.

A filter or membrane technique for obtaining the cell juxtaposition and membrane contact required for electrofusion has also been outlined in the literature. In this approach, the cells to be fused are deposited in the pores of filters or artificial membranes by suction or by binding the cells to the surface of artificial membranes by electrostatic attraction. The filters or membranes are placed between parallel electrodes and the distance between the filters or membranes is reduced until contact is made between the cells on the opposing filters or membranes. Zimmermann, U., "Electrical Breakdown, Electropermeabilization and Electrofusion," Rev. Physiol. Biochem. Pharmacol., 150, 175-256 (1986). This approach, of course, is limited to cell-to-cell electrofusion in vitro.

Other suggestions for achieving the cell contact required for in vitro electrofusion include: (1) use of crossed magnetic fields and magnetically-coated cells; (2) use of sonic fields created by a piezoelectric crystal; (3) use of laser beams; and (4) use of specific chemical reactions such as avidin-biotin binding and cross-linking agents. Zimmermann, U., "Electrical Breakdown, Electropermeabilization and Electrofusion," Rev. Physiol. Biochem. Pharmacol., 105, pp. 175-256 (1986); Wojchowski, D. M. and Sytkowski, A. J., "Hybridoma Production by Simplified.

Avidin-Mediated Electrofusion," J. Immunol. Meth., 90, pp. 173-77 (1986); Schierenberg, E., "Laser-Induced Cell Fusion," Cell Fusion (Ed., Arthur E. Sowers), pp. 409-18 (Plenum Press, N.Y. 1987).

Reported attempts to electrofuse cells in vitro without employing a non-uniform alternating current field to align the cells and form pearl chains have met with some success. For some of these approaches (i.e., magnetic, acoustic, laser and membrane or filter electrofusion techniques), a great deal of work must be done before it can be determined whether the techniques are practical, efficient and susceptible of general in vitro application. The remaining reported approaches are subject to important restrictions and limitations that make it unlikely they will replace the dielectrophoresis method as the method of choice for general in vitro application.

The electrofusion methods described in the literature, including the dielectrophoresis approach, are directed to the fusion of discrete biological particles to other discrete biological particles in vitro. All of these methods are characterized by significant limitations and disadvantages. A need therefore exists for methods that avoid these limitations and disadvantages and permit in vitro, in situ or in vivo electrofusion of biological particles to cells of animal or plant tissues.

Gonorrhea is a host-specific affliction caused by the human bacterial pathogen Neisseria gonorrhoeae ("N.

gonorrhoeae"). Pathogenesis of the disease is initiated when N. gonorrhoeae binds to specific gonococcal attachment receptors found exclusively on the surface of membranes of human cells. Many attempts have been made to infect animals with this human pathogen since Neisser first described the organism in 1879. None of those attempts, however, have produced a practical, biologically-relevant animal model that produces reproducible gonococcal lesions.

Early attempts to create a laboratory model for gonococcal infections were surveyed by Hill in 1944. Hill, J. H., Amer. J. Syphilis, 58, pp. 471-510 (1944). More recent reports on attempts to create such a model were reviewed by Kraus in 1977. Kraus, J., The Gonococcus (Ed., R. B. Roberts), pp. 415-31 (John Wiley & Sons, N.Y. 1977). Attempts to create laboratory models for gonorrhea have been based on gonococcal infection of chimpanzees to produce urethritis and pharyngitis (Lucas, C. T., et. al., J. Am. Med. Assoc., 216, pp. 1612-14 (1971); Arko, R. J., Science, 177, pp. 1200-01 (1972); Arko, R. J., et al., J. Infect. Dis., 130, pp. 160-64 (1976); Arko, R. J., et al., J. Infect. Dis., 133, pp. 441-447 (1976); Brown, W. J., et al., Br. J. Vener. Dis., 48, pp. 177-78 (1972); Kraus, S. J., et al., J. Clin. Invest., 55, pp. 1349-56 (1975)), infection of animals through subcutaneous tissue cavity chambers (Arko, R. J., Lab Animal Sci., 23, pp. 105-106 (1973); Veale, D. R., J. Med. Microbiol., 8, pp. 325-35 (1975)), and infection of chorioallantoic membranes and other tissues of chick embryos.

(Bushanan, T. M. and Gotschlich, E. C., J. Exp. Med., 137, pp. 196-200 (1973); Shcherbakova, N. I., et al., Zh. Mikrobiol. Epidemiol. Immunobiol., 4, pp. 37-40 (1986)). Attempts to produce infections in the anterior chamber of the eye, the brain and the peritoneal cavity of animals have also been described (Miller, C. P., et al., J. Inf. Dis., 77, pp. 192-200 (1945); Diena, B. B., et al., Br. J. Vener. Dis., 51, pp. 22-24 (1975)). Kita recently reported that mice treated with estradiol are susceptible to disseminated gonococcal infection produced by intraperitoneal injection of N. gonorrhoeae 57-120, apparently because the steroid hormone impairs PMN bacteriocidal activity. Kita, E., et al., Infect. Immun., 49, pp. 238-243 (1985). Kita has also reported the vaginal infection of ddY mice with N. gonorrhoeae PH2. Kita, E., et al., J. Infect. Dis., 143, pp. 67-70 (1981); Kita, E. and Kashiba, S., Br. J. Vener. Dis., 60, pp. 219-25 (1984).

All of these reported attempts to produce animal models for gonococcal infection are characterized by significant drawbacks and limitations. Moreover, investigators in other laboratories have been unable to obtain reproducible lesions with many of these infectivity models. The chimpanzee models are impractical because the animals are a threatened species, are in limited supply and are extremely expensive. The utility of other models is limited because infection occurs at anatomical sites that are not normally infected by N. gonorrhoeae in humans, and because lesions are produced

within tissues instead of on mucosal epithelial surfaces where gonococcal infections occur naturally in humans. The vaginal infection of mice reported by Kita is marked by serious limitations that severely limit the utility of the model for most practical research applications. Infection apparently can be produced only with the PH2 strain of N. gonorrhoeae in only ddY mice, and only during a brief preovulatory period in the estrous cycle.

To date, investigators have been unable to overcome the problem that the cells of animals other than humans simply lack the specific human gonococcal receptors required for the initiation of clinically-meaningful pathogenesis. A need therefore exists for practical, clinically-relevant animal models for human receptor-mediated gonorrhea.

Disclosure of Invention

The present invention relates to novel methods for electrofusing biological particles to cells of animal or plant tissues in vitro, in situ or in vivo. Selected biological particles are placed in contact with cells of a selected tissue. The biological particles are fused to cells of the tissue by means of an electric field. By virtue of this invention, animals and plants characterized by features other than those typical of the native species may be produced.

The methods of this invention may be used to produce animal or plant models for the study of receptor-mediated

processes. One embodiment has been used to produce an animal model for the study of ocular gonorrhea by electrofusing human cells, with receptors for the human bacterial pathogen Neisseria gonorrhoeae, to epithelial cells in the histologically intact superficial corneal tissue of living rabbits. Gonococcal adherence assays, specificity studies based on scanning electron microscopy techniques and production of purulent ocular gonorrhea in vivo have demonstrated that functional human gonococcal attachment receptors were electrofused to cells of the corneal epithelium of the rabbits.

The methods of this invention may be used for other applications where it is advantageous to fuse selected cells, liposomes or other biological particles to cells of plant or animal tissues in vitro, in situ or in vivo. The methods of this invention are safe, simple, practical and efficient, and avoid limitations and disadvantages that characterize coventional in vitro cell-to-cell electrofusion techniques.

Best Modes for Carrying Out the Invention

This invention relates to methods for electrofusing biological particles to cells of animal or plant tissues. The methods of the invention may be used for in vitro electrofusion of biological particles to cells of tissues that have been excised from an animal or plant, in situ electrofusion of biological particles to cells of tissues in dead animals or plants, or in vivo electrofusion of

biological particles to cells of tissues in living animals or plants.

The methods of this invention generally comprise the following steps: (1) biological particles and animal or plant tissue are selected and prepared for electrofusion; (2) the biological particles are placed in contact with cells of the selected tissue; and (3) the biological particles are fused to cells of the tissue by subjecting the biological particles and cells of the tissue to an electric field created by application of one or more pulses of electric current.

Biological particles that may be selected for electrofusion to cells in animal or plant tissue in accordance with the methods of this invention include animal cells, plant cells, microorganisms such as bacteria and yeast cells, liposomes, cell vesicles and cell organelles such as lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and other vacuoles. Some of these biological particles, e.g., plant cells, bacteria and yeast cells, have cell walls that are known to interfere with electrofusion. Prior to electrofusion according to the methods of this invention, the membranes of such particles are exposed by stripping or removing the cell walls by conventional enzymatic techniques or other known methods. Zimmermann, U., "Electrical Breakdown, Electropermeabilization, and Electrofusion," Rev. Physiol. Biochem. Pharmacol., 105, pp. 175-256 (1986).

Other general steps necessary for the preparation of selected biological particles for electrofusion in accordance with the methods of this invention depend on the means by which the particles are to be placed in contact with cells of the selected tissue. Those steps are described below in connection with means that may be employed to place selected biological particles in contact with cells of a selected tissue in the methods of this invention.

The methods of this invention may advantageously be used to fuse biological particles to any animal or plant tissue that may be exposed or excised so that biological particles may be placed in intimate contact with the membranes of the cells in the tissue. Biological particles may be fused to histologically intact tissues or to tissues that have been treated, manipulated or modified prior to electrofusion. Cell walls of cells in plant tissues are stripped or removed using conventional techniques prior to electrofusion in accordance with this invention. For in vitro applications, the selected tissue is, of course, excised from a donor plant or animal using known procedures. If the tissue is not used immediately after collection, appropriate storage media are employed to preserve and maintain the integrity of the excised tissue. Tissues used in accordance with in vitro, in situ or in vivo applications of the methods of this invention are preferably rinsed free of debris with a physiologically compatible buffered isotonic solution such as phosphate-buffered saline ("PBS") solution, before selected

biological particles are placed in contact with and electrofused to cells of the selected tissue. The pH of the solution is adjusted in accordance with known techniques so that it is compatible with the tissue selected for electrofusion.

Other general steps necessary for the preparation of tissues selected for electrofusion according to the methods of this invention depend on the means by which the particles are to be placed in contact with cells of the tissue. Those steps are described below in connection with means that may be employed to place biological particles in contact with cells of a selected tissue.

In known in vitro cell-to-cell fusion methods, the cell juxtaposition and membrane contact required for electrofusion is commonly obtained by subjecting the cell suspension to an inhomogenous alternating current field that causes the cells to align and form strings of contiguous cells known as pearl chains by the process of dielectrophoresis. Although use of an inhomogeneous alternating current field so far has proven to be the most practical and efficient means of achieving the contact required for in vitro cell-to-cell electrofusion, the alternating current field itself creates problems and is marked by disadvantages that limit the utility of known methods. Those problems and limitations are discussed above in connection with the description of the background of this invention. According to the methods of this invention, biological particles may be fused to cells of tissues without

utilizing the dielectrophoresis step required in conventional in vitro cell-to-cell electrofusion methods. Thus, this invention avoids disadvantages and limitations of conventional cell-to-cell in vitro processes.

In accordance with the methods of this invention, the contact required for electrofusion is preferably obtained by applying a mechanical force sufficient to cause intimate contact between selected biological particles and cells of a selected tissue. In a preferred embodiment of this invention, the contact required for electrofusion is achieved by depositing the particles by centrifugation onto a support such as a filter (e.g., Millipore HAWP filters (Millipore; New Bedford, MA)) or an electrostatically charged disc (e.g., Zetaprobe blotting membrane disc (Bio-Rad; Richmond, CA)) in an evenly-distributed layer. The particle-laden support is placed cell-side down on the surface of a selected tissue. A "fusion electrode" connected to a direct current pulse generator is positioned on the upper surface of the support. Another electrode ("ground electrode") connected to the direct current pulse generator is grounded to the tissue or plant or animal containing the tissue. The fusion electrode is pressed down on the support with a force sufficient to create contact between the particles on the support and cells in the tissue. Particles are fused to cells of the tissue by applying direct current pulses to the fusion electrode. After removing the fusion electrode, ground electrode and support, the tissue is washed with a buffered isotonic

solution such as PBS or Hank's BSS to remove any unfused particles.

Optimal conditions for electrofusion using mechanical force to create contact between the particles and cells of the selected tissue in any particular case may depend on the nature of the selected particles and tissue, the preparation and deposition of the particles on the support, the configuration of the fusion electrode and perhaps other factors. Such optimization may be achieved using conventional methods and means that are known to those of skill in the art.

For example, human HL60 or U937 lymphoma cells deposited by centrifugation in an even layer on filters or electrostatically-charged discs may be electrofused to the histologically intact corneal epithelium of a rabbit in vitro, in situ or in vivo, by applying with an eye-shaped electrode a mechanical force on the surface of the filter or disc sufficient to create a pressure of about 600 to 800 g/cm^2. Fusion is preferably accomplished under constant voltage conditions by applying to the electrode 3 square-wave 20 microsecond pulses of direct current with an amplitude of 20 volts at a pulse rate of 1 pulse per second. The current, which may be calculated using Ohm's law, depends on the electrical resistance of the tissue, which is about 3000 ohms for in vivo applications.

In general, biological particles may be placed in contact with cells of a selected tissue and electrofused in

accordance with the methods of this invention by applying mechanical forces that range from those that create slight contact, e.g., about 15 g/cm^2 of pressure on the upper surface of the support, to those that generate pressures of about 1500 g/cm^2 on the upper surface of the support. At the low end of this range, the only requirement is that the force be sufficient to create contact between the biological particles and cells of the selected tissue. The limit at the high end of this range is that the force must not be so great that it causes damage to the particles or tissue selected for electrofusion.

Electrical parameters for electrofusion in accordance with this as well as other embodiments of the methods of this invention may also be varied. In this connection, electrofusion may be carried out with direct current fields created by applying one or more pulses of direct current to the fusion electrode using a range of voltages and currents sufficient to cause fusion, but not so high that the biological particles or tissues are damaged. The number, duration and pulse rate of the direct current pulses may also be varied.

It may be advantageous to employ a fusion electrode adapted to conform to the surface of the selected tissue. For example, a fusion electrode with a surface that matches the curvature of a rabbit eye may be used when fusing human cells to the corneal epithelia of anesthetized rabbits.

It should be noted that the steps of this and other embodiments of this invention may be repeated to successively electrofuse any desired number or kind of selected biological particles to cells of a selected tissue. It should also be noted that in this and other embodiments of this invention, it is not essential to place the biological particles in contact with cells of the selected tissue before applying the fusion pulse or pulses. Thus, electrofusion may be carried out by exposing selected biological particles and cells of the selected tissue to an electric field sufficient to render the particles and cells of the tissue "fusogenic" before placing the particles in contact with cells of the tissue.

The contact required for electrofusion in accordance with the methods of this invention may be obtained by means other than the application of a mechanical force. Contact between selected particles and cells of a selected tissue may be obtained, for example, by binding to the particles antibodies exhibiting specificity to cells of the selected tissue. Particles coated with such antibodies are strongly bound to cells of the selected tissue when the antigen-combining sites of the antibody bind with cells in the tissue. Human HL60 cells, for example, possess antibody receptor sites (Fc receptors) that bind IgG in a manner that leaves the antigen-combining sites of the immunoglobulins pointed away from the surface of the cell. The immunoglobulins on the surface of the HL60 cells remain free to bind with specific antigens. Contact between the HL60 cells and cells of a

selected tissue may be achieved by coating the HL60 cells with immunoglobulins that exhibit specificity to cells of the selected tissue. When the HL60 cells coated with IgG are introduced to the tissue, the HL60 cells are strongly bound to the cells in the tissue by the antibody.

Particles coated with antibodies may be introduced to a selected tissue in various ways. Cells coated with IgG, for example, may be injected into an animal by intravenous injection. After the immunoglobulins on the coated cells bind to cells in the target tissue, the coated cells may be fused to cells of the tissue by applying a pulsed electric field. Cells coated with antibodies may also be placed in contact with a selected tissue by depositing the coated cells on filters or membranes by centrifugation or suction, placing the filter or membrane cell-side down on the tissue and applying mechanical force on the upper surface of the filter or membrane. Very specific fusion products may be efficiently obtained in this way.

Specific chemical reactions may also be employed to obtain the contact between selected particles and cells of a selected tissue that is required for electrofusion in accordance with the methods of this invention. Photoreactive bifunctional cross-linking agents such as N-Hydroxysuccinimidyl 4-azidobenzoate, for example, may be incubated in the dark with selected cells or liposomes to covalently link one end of the linear cross-linking agent to proteins on the cells or liposomes. Cells or liposomes bound to the

cross-linking agent are then introduced to cells of the selected tissue and exposed to high intensity light so that the photoreactive end of the cross-linking agent binds to proteins on cells of the selected tissue by photolysis, firmly binding the cells or liposomes to cells in the selected tissue. After contact between the cells or liposomes and cells of the selected tissue is achieved with the bifunctional cross-linking agent, fusion is accomplished by applying an electric field. Other chemical reactions that may be employed to obtain the contact required for electrofusion in accordance with the methods of this invention include the use of an avidin-biotin system, where avidin is used to mediate the attachment of biotinylated cells to biotinylated tissues or to biotinylated immunogens that exhibit specificity and bind firmly to cells of the tissue selected for electrofusion.

The biological particle-tissue contact required for electrofusion in accordance with this invention may also be obtained by centrifugation. Biological particles in suspension may be deposited on a selected tissue and placed in contact with cells of the tissue by centrifugation. Fusion may be accomplished by exposing the particles and cells of the tissue to an electric field to render them fusogenic before the particles are deposited on the tissue by centrifugation, or by subjecting the particles and cells of the tissue to an electric field after the particles are placed in contact with cells in the tissue by centrifugation.

The requisite contact between selected biological particles and cells of a selected tissue is obtained in another embodiment of this invention by means of a magnetic field. Magnetically-coated particles are placed in contact with cells of a selected tissue by exposing the coated particles to a magnetic field with a gradient oriented in a direction that causes the particles to move toward the surface of the tissue. The force of the field is sufficient to create close contact between the particles and cells in the tissue.

It should be understood that the biological particle-tissue contact required for electrofusion in accordance with the methods of this invention may also be obtained by using a combination of means. Particle-tissue contact may be achieved, for example, by combining bifunctional cross-linking agents, centrifugation and application of a mechanical force to achieve intimate contact between selected biological particles and cells of a selected tissue.

Biological particles selected for electrofusion in accordance with the methods of this invention may be modified or treated according to known procedures before being placed in contact with cells of a selected tissue. Cells selected for electrofusion, for example, may be infected with microorganisms that bind to the surface of the cell membrane or with organisms that are taken up by the cell and survive as intracellular parasites. Certain viruses, bacteria,

protozoa and mycotic agents may all be used as pathogens. Cells may be treated with drugs or biological response modifiers such as lymphokines or monokines, or transfected with isolated genes or other DNA before being electrofused to cells of a selected tissue. In addition, specific receptor molecules may be covalently bound to the surface of selected cells prior to electrofusion. Bifunctional double cross-linking agents such as N-hydroxysuccinimidyl 4-azidobenzoate, for example, may be used to covalently bind microbial or toxin attachment components to cells before the cells are placed in contact with cells of a selected tissue for electrofusion. Selected cells may also be fused with the same or other kinds of cells before being electrofused to cells in a selected tissue.

Similarly, liposomes may be employed as carriers for drugs, covalently-linked receptor molecules, immunoregulatory agents, cloned genes and other agents. Liposomes carrying anti-tumor drugs, for example, may be directly electrofused to neo-plastic tissue. Liposomes, which are generally non-immunogenic, may be used to avoid rejection problems that arise as a result of the functional immune response of an animal host.

In accordance with the methods of this invention, drugs, genes and other molecules (e.g., microbial, toxin and hormone receptors) may be bound to or incorporated in a host animal's own leukocytes or erythrocytes before the leukocytes or erythrocytes are electrofused to cells of a selected tissue

of the host animal. If the material bound or incorporated to the leukocytes or erythrocytes is not itself antigenic, the autograft created by electrofusion will not be rejected by the immune system of the host animal.

One embodiment of the methods of this invention has been used to produce a novel animal model for the study of ocular gonorrhea. Functional gonococcal attachment receptors from the surface membranes of human U937 and HL60 cells were transferred to epithelial cells in the histologically intact superficial corneal tissue of living rabbits by electrofusion. Acute purulent gonococcal keratoconjunctivitis was observed in the eyes of the rabbits after infecting the modified rabbit corneal tissue with the human bacterial pathogen Neisseria gonorrhoeae. The example below is set forth in order that this invention may be more fully understood. This example is for the purpose of illustration only and it is not to be construed as limiting the scope of this invention in any way.

EXAMPLE

Nonadherent human HL60 promyelocytic leukemia cells (ATCC CCL 240; American Type Culture Collection, Rockville, MD) and nonadherent murine WEHI-3 myelocytic leukemia cells (ATCC TIB 68; American Type Culture Collection, Rockville, MD) were prepared for electrofusion by growing the cells in suspension cultures at 37°C in Costar plastic tissue culture flasks containing DMEM (GIBCO; Grand Island, NY) supplemented with

10% fetal bovine serum (Flow Laboratories; McLean, VA) in an atmosphere of 95% air and 5% CO_2. Adherent rabbit skin cells (Smith, C.A. and Lanca, G.J., Arch. Virol., 74, pp. 311-23 (1982)) obtained from Dr. G.J. Lanca, Department of Medical Microbiology and Immunology at the University of South Florida College of Medicine, Tampa, Florida, were prepared for electrofusion by growing cells in monolayer cultures at 37°C in plastic tissue culture flasks containing Eagle's MEM (GIBCO; Grand Island, NY) supplemented with 5% newborn bovine calf serum (Flow Laboratories; McLean, VA). The adherent rabbit skin cells were detached from the plastic flasks with 0.25% trypsin (GIBCO; Grand Island, NY). Prior to electrofusion, suspensions of HL60, WEHI-3 and rabbit skin cells were washed twice by centrifugation (200xg, 4°C, 10 min) in phosphate-buffered saline solution (pH 7.4; 150 mM NaCl; 3.4 mM KCl; 10.1 mM Na_2HPO_4; and 1.8 mM KH_2PO_4). The final washed sedimented pellets of HL60, WEHI-3 and rabbit skin cells were resuspended in the same phosphate-buffered saline solution at cell densities of about 10^8 to 10^9 per ml.

Four adult New Zealand white rabbits (Cummings Rabbitry; Lutz, FL) (3-4 kg) housed, fed, watered and handled in compliance with NIH regulations (Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23 (Washington, D.C. 1985)) were prepared for electrofusion by intramuscular injection of 100 mg/ml ketamine-HCl (Bristol Laboratories; Syracuse, NY) containing 1 mg/ml acepromazine maleate (Med-Tech, Inc.; Elwood, KS) at a dosage of 15 mg

ketamine-HCl/kg body weight. After the rabbits were tranquilized, anesthesia was induced by administering medical/dental grade N_2O (Puritan Bennett; Gonzales, FL) through a nose cone until no ocular muscle responses occurred when the eyes were flushed with PBS. Effective anesthesia was maintained throughout the electrofusion procedure by repeated interrupted inhalation of N_2O for 5 seconds administered intermittently at 10 second intervals. The anesthetized rabbits were allowed to breathe room air normally during the intervening 5 second periods. The rabbits recovered from anesthesia within approximately 1 minute after their last exposure to N_2O.

HL60 cells, murine WEHI-3 cells and rabbit skin cells were electrofused to the intact superficial corneal epithelial tissue of the anesthetized rabbits as summarized below in Table 1. Electrofusion was carried out by separately collecting washed HL60 cells, murine WEHI-3 cells and rabbit skin cells by centrifugation (200xg, 4°C, 10 min) on HAWP 8mm diameter Millipore filters (Millipore; New Bedford, MA) positioned at the bottom of centrifuge tubes. After discarding the supernatants, the filters were removed with forceps and placed cell-side down on the PBS-washed corneal surfaces of the anesthetized rabbits.

A concave titanium electrode, housed in an insulating handle and machined to reflect the radius of curvature of the rabbit corneal surfaces, was positioned on the upper side of the filters. Another electrode was attached to the buccal

mucosa of the rabbits. Leads from a direct current pulse generator were connected to both electrodes; electrical resistance between the electrodes measured about 3000 ohms.

The eye-shaped titanium fusion electrode positioned on the upper side of the filter was pressed manually with a force sufficient to create a pressure of about 600 to 800 g/cm^2 on the upper surface of the filter. Fusion was accomplished under constant voltage conditions by applying to the fusion electrode 3 square-wave 20 microsecond pulses of direct current with an amplitude of 20 volts at a pulse rate of 1 pulse per second. After fusion pulses had been applied, the fusion electrode, ground electrode and filters were removed from the rabbits. The corneal tissue of the rabbits was then thoroughly washed with PBS to remove any unfused cells.

Within one hour after applying fusion pulses, the eyes of the rabbits were infected with a thick suspension of viable N. gonorrhoeae Pgh 3-2 (J.C. McMichael, ImmunoMed Corporation; Tampa, FL) using a Pasteur pipette. After 10 minutes the eyes were thoroughly washed free of any unbound bacteria with PBS dispensed from a squeeze bottle. Infection was carried out with N. gonorrhoeae Pgh supplemented with 1% Isovitalex (DIFCO; Detroit, MI) in an atmosphere of 95% air plus 5% CO_2. Gonococcal cell suspensions (about 10^8 colony forming units per ml) were prepared by flooding plates containing fresh overnight stable type 2 colonies with

modified M-K medium (McCarey, B. E., et. al., Ann. Ophthalmol., 8, pp. 1488-92 (1976)).

The design and results of this example are summarized below in Table 1.

TABLE 1
PURULENT KERATOCONJUNCTIVITIS MEDIATED BY HUMAN RECEPTORS IN A RABBIT MODEL FOR OCULAR GONORRHEA

DESIGN[a]

Treatment of Corneas	Rabbit #1 Left	Rabbit #1 Right	Rabbit #2 Left	Rabbit #2 Right	Rabbit #3 Left	Rabbit #3 Right	Rabbit #4 Left	Rabbit #4 Right
Human HL60 Cells	-	+	+	+	-	-	-	-
Mouse WEHI-3 Cells	-	-	-	-	+	+	-	-
Rabbit Skin Cells	-	-	-	-	-	-	+	+
Fusion Pulses	+	-	+	+	+	+	+	+
N. gonorrhoeae Pgh 3-2	+	+	-	+	-	+	-	+
CLINICAL EVALUATION[b] 3 HR POSTINFECTION								
Injection	-	-	-	+	-	-	-	-
Chemosis	-	-	-	++	-	-	-	-
Exudate	-	-	-	+	-	-	-	-
CLINICAL EVALUATION[b] 6 HR POSTINFECTION								
Injection	-	-	-	++	-	-	-	-
Chemosis	-	+/-	-	++	-	-	-	-
Exudate	-	+/-	-	++	-	-	-	-

a Pluses and minuses indicate whether treatments were (+) or were not (-) performed on the respective left and right corneas of the 4 rabbits.

b Pluses and minuses indicate postinfection observations made in accordance with the following grading scheme for assessing the severity of ocular gonorrhea: no symptoms (-); trace (+/-); slight (+); mild (++); moderate (+++); and severe (++++).

The top portion of Table 1 depicts the design of this example. Rabbit #1 served as a control animal. Fusion pulses were applied to the left cornea of rabbit #1 in the absence of HL60, WEHI-3 or rabbit skin cells. Human HL60 cells were placed on the right cornea of rabbit #1, but no fusion pulses were applied. Human HL60 cells, mouse WEHI-3 cells and rabbit skin cells were respectively electrofused to both eyes of rabbits #2, #3, and #4. Unfused cells were removed by washing the eyes with sterile PBS. Both eyes of rabbit #1, but only the right eyes of rabbits #2, #3, and #4 were infected with N. gonorrhoeae Pgh 3-2. Gonococci that did not attach to the cornea were removed by rinsing the eyes with sterile PBS. Both eyes of all four of the rabbits appeared normal immediately after they had been infected with bacteria and rinsed free of unattached gonococci.

The eyes of all four of the rabbits were clinically evaluated for three symptoms of purulent gonococcal keratoconjunctivitis at 3 and 6 hours after infection. Injection, chemosis and exudate formation were scored using the plus/minus scheme described in Table 1. The lower portions of Table 1 report clinical evaluation data taken at 3 and 6 hours after infection. These data show that purulent gonococcal lesions occurred only in the right eye of rabbit #2, which had been electrofused with human HL60 cells having receptors for the human pathogen N. gonorrhoeae. Ocular gonorrhea did not develop in the infected right eyes of rabbits that had been electrofused with mouse WEHI-3 cells or

rabbit skin cells that lack receptors for the bacterium. Taken together, these data demonstrate that the method of this invention may be used to create an animal model for ocular gonorrhea by electrofusing human cells with receptors for the human bacterial pathogen Neisseria gonorrhoeae to epithelial cells in the histologically intact superficial corneal tissue of living rabbits.

The foregoing example demonstrates that the methods of this invention may be used to produce a clinically-relevant animal model for ocular gonorrhea. It should be understood, however, that the methods of this invention may be used for many other applications. The methods may be used, for example, to create other animal models for the study of the pathogenesis of infectious diseases important in human and veterinary medicine where infection is initiated by the binding of the etiological agent directly to host or tissue-specific receptors. Such diseases include, but are not limited to, acquired immune deficiency syndrome (AIDS) caused by HIV infections, swine rhinitis and sheep pneumonia caused by mycoplasmae infections, and other diseases caused by rhino viruses or corona viruses. Animal models for such diseases may be created by transferring appropriate attachment receptors to cells of selected tissues of common laboratory animals by electrofusion in vivo.

Other applications of the methods of this invention include site-specific delivery of drugs or other agents for

chemotherapy, immunotherapy and replacement therapy to manage or control a variety of experimental, human and veterinary disorders. Liposomes carrying potent antitumor agents, for example, may be directly fused to cells in neoplastic tissue in accordance with this invention. Site-specific delivery of the antitumor agents achieves a high concentration of the drug where it is needed most and minimizes or avoids undesirable side effects that are normally encountered when the drug is administered systemically.

The methods of this invention may also be used in gene therapy to correct inherited genetic disorders. Genetic disorders caused by defective genes that result in metabolic deficiencies, for example, may be treated by electrofusing cells or liposomes containing functional genes that code for a particular enzyme directly to cells in the afflicted animal.

In general, the methods of this invention may be used for any application where it is advantageous to fuse selected biological particles to cells of selected tissues of plants or animals.

Having described a number of embodiments of the methods of this invention, the fusion products, the animal and plant models, and the animals and plants that may be produced with the methods of this invention, it is apparent to those skilled in the art to which the invention pertains that

various changes and modifications may be made without departing from the spirit and scope of the invention as defined by the claims appended hereto.

WHAT IS CLAIMED IS:

1. A method for fusing biological particles to cells of animal or plant tissue comprising the steps of:

a. selecting biological particles to be fused to cells of animal or plant tissue;

b. selecting animal or plant tissue;

c. placing the selected biological particles in contact with cells of the selected tissue; and

d. subjecting the selected biological particles and cells of the selected tissue to an electric field.

2. The method of claim 1, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

3. The method of claim 1, wherein the selected animal or plant tissue comprises histologically intact animal or plant tissue.

4. The method of claim 1, wherein the selected biological particles are placed in contact with cells of the selected tissue by means of a mechanical force.

5. The method of claim 4, wherein the mechanical force is applied with an electrode juxtaposed with the selected biological particles and selected tissue.

6. The method of claim 1, wherein the electric field comprises a direct current electric field.

7. The method of claim 6, wherein the direct current field is created by applying a pulse of direct current to an electrode juxtaposed with the selected biological particles and selected tissue.

8. The method of claim 6, wherein the direct current field is created by applying multiple pulses of direct current to an electrode juxtaposed with the selected biological particles and selected tissue.

9. A method for fusing biological particles to cells of animal or plant tissue comprising the steps of:

a. selecting biological particles to be fused to cells of animal or plant tissue;

b. selecting a support;

c. depositing the selected biological particles on the selected support;

d. selecting animal or plant tissue;

e. placing the selected support on the selected tissue to juxtapose the selected biological particles deposited on the support with cells of the selected tissue;

f. placing the biological particles deposited on the selected support in contact with cells of the selected tissue by means of a mechanical force applied to a surface of the support; and

g. subjecting the selected biological particles and cells of the selected tissue to an electric field.

10. The method of claim 9, wherein the biological particles are selected from the group consisting of animal

cells, plants cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

11. The method of claim 9, wherein the support is selected from the group consisting of filter paper and electrostatically-charged discs.

12. The method of claim 9, wherein the selected biological particles are deposited on the selected support by means of centrifugation.

13. The method of claim 9, wherein the selected animal or plant tissue comprises histologically intact animal or plant tissue.

14. The method of claim 9, wherein the mechanical force is applied with an electrode positioned on a surface of the support.

15. The method of claim 9, wherein the electric field comprises a direct current electric field.

16. The method of claim 15, wherein the direct current field is created by applying a pulse of direct current to an electrode positioned on a surface of the selected support.

17. The method of claim 15, wherein the direct current field is created by applying multiple pulses of direct current to an electrode positioned on a surface of the selected support.

18. A method for producing animals or plants characterized by features that differ from those typically displayed by the native species comprising the steps of:

a. selecting biological particles;

b. selecting an animal or plant having at least one tissue;

c. placing the selected biological particles in contact with cells of a tissue of the selected animal or plant; and

d. subjecting the selected biological particles and cells of the tissue of the selected animal or plant to an electric field.

19. The method of claim 18, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

20. The method of claim 18, wherein the tissue of the selected animal or plant comprises histologically intact animal or plant tissue.

21. The method of claim 18, wherein the selected biological particles are placed in contact with cells of the tissue of the selected animal or plant by means of a mechanical force.

22. The method of claim 21, wherein the mechanical force is applied with an electrode juxtaposed with the selected

biological particles and cells of the tissue of the selected animal or plant.

23. The method of claim 18, wherein the electric field comprises a direct current electric field.

24. The method of claim 23, wherein the direct current field is created by applying a pulse of direct current to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal or plant.

25. The method of claim 23, wherein the direct current field is created by applying multiple pulses of direct current to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal or plant.

26. A method for producing animals or plants characterized by features that differ from those typically displayed by the native species comprising the steps of:

a. selecting biological particles;

b. selecting a support;

c. depositing the selected biological particles on the selected support;

d. selecting an animal or plant having at least one tissue;

e. placing the selected support on a tissue of the selected animal or plant to juxtapose the selected biological particles deposited on the support with cells of the tissue of the selected animal or plant;

f. placing the biological particles deposited on the selected support in contact with cells of the tissue of the selected animal or plant by means of a mechanical force applied to a surface of the support; and

g. subjecting the selected biological particles and cells of the tissue of the selected animal or plant to an electric field.

27. The method of claim 26, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

28. The method of claim 26, wherein the support is selected from the group consisting of filter paper and electrostatically-charged discs.

29. The method of claim 26, wherein the selected biological particles are deposited on the selected support by means of centrifugation.

30. The method of claim 26, wherein the tissue of the selected animal or plant comprises histologically intact animal or plant tissue.

31. The method of claim 26, wherein the mechanical force is applied with an electrode positioned on a surface of the support.

32. The method of claim 26, wherein the electric field comprises a direct current electric field.

33. The method of claim 32, wherein the direct current field is created by applying a pulse of direct current to an electrode positioned on a surface of the selected support.

34. The method of claim 32, wherein the direct current field is created by applying multiple pulses of direct current to an electrode positioned on a surface of the selected support.

35. A method for producing animal or plant models for receptor-mediated pathogenic processes comprising the steps of:

a. selecting biological particles with functional receptors for a pathogen;

b. selecting an animal or plant having at least one tissue;

c. placing the selected biological particles in contact with cells of a tissue of the selected animal or plant; and

d. subjecting the selected biological particles and cells of the tissue of the selected animal or plant to an electric field.

36. The method of claim 35, further comprising the step of infecting the selected biological particles with the pathogen.

37. The method of claim 35 or 36, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes,

phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

38. The method of claim 35 or 36, wherein the tissue of the selected animal or plant comprises histologically intact animal or plant tissue.

39. The method of claim 35 or 36, wherein the selected biological particles are placed in contact with cells of the tissue of the selected animal or plant by means of a mechanical force.

40. The method of claim 39, wherein the mechanical force is applied with an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal or plant.

41. The method of claim 35 or 36, wherein the electric field comprises a direct current electric field.

42. The method of claim 41, wherein the direct current field is created by applying a pulse of direct current to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal or plant.

43. The method of claim 41, wherein the direct current field is created by applying multiple pulses of direct current to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal or plant.

44. A method for producing animal or plant models for receptor-mediated pathogenic processes comprising the steps of:

a. selecting biological particles with functional receptors for a pathogen;

b. selecting a support;

c. depositing the selected biological particles on the selected support;

d. selecting an animal or plant having at least one tissue;

e. placing the selected support on a tissue of the selected animal or plant to juxtapose the selected biological particles deposited on the support with cells of the tissue of the selected animal or plant;

f. placing the biological particles deposited on the selected support in contact with cells of the tissue of the selected animal or plant by means of a mechanical force applied to a surface of the support; and

g. subjecting the selected biological particles and cells of the tissue of the selected animal or plant to an electric field.

45. The method of claim 44, further comprising the step of infecting the selected biological particles with the pathogen.

46. The method of claim 44 or 45, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

47. The method of claim 44 or 45, wherein the support is selected from the group consisting of filter paper and electrostatically-charged discs.

48. The method of claim 44 or 45, wherein the selected biological group particles are deposited on the selected support by means of centrifugation.

49. The method of claim 44 or 45, wherein the tissue of the selected animal or plant comprises histologically intact animal or plant tissue.

50. The method of claim 44 or 45, wherein the mechanical force is applied with an electrode positioned on a surface of the support.

51. The method of claim 44 or 45, wherein the electric field comprises a direct current electric field.

52. The method of claim 51, wherein the direct current field is created by applying a pulse of direct current to an electrode positioned on a surface of the selected support.

53. The method of claim 51, wherein the direct current field is created by applying multiple pulses of direct current to an electrode positioned on a surface of the selected support.

54. A method for producing animal models for gonorrhea comprising the steps of:

a. selecting biological particles with functional receptors for Neisseria gonorrhoeae;

b. selecting an animal having at least one tissue;

c. placing the selected biological particles in contact with cells of a tissue of the selected animal; and

d. subjecting the selected biological particles and cells of the tissue of the selected animal to an electric field.

55. The method of claim 54, further comprising the step of infecting the selected biological particles with Neisseria gonorrhoeae.

56. The method of claim 54 or 55, wherein the biological particles are selected from the group consisting of human HL60 or U937 lymphoma cells.

57. The method of claim 54 or 55, wherein the selected animal is a rabbit.

58. The method of claim 54 or 55, wherein the tissue of the selected animal comprises corneal tissue.

59. The method of claim 54 or 55, wherein the tissue of the selected animal comprises histologically intact superficial corneal epithelial tissue.

60. The method of claim 54 or 55, wherein the selected biological particles are placed in contact with cells of the tissue of the selected animal by means of a mechanical force.

61. The method of claim 60, wherein the mechanical force is applied with an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal.

62. The method of claim 54 or 55, wherein the electric field comprises a direct current electric field.

63. The method of claim 62, wherein the direct current field is created by applying a pulse of direct current to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal.

64. The method of claim 62, wherein the direct current field is created by applying multiple pulses of direct current to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal.

65. A method for producing animal models for gonorrhea comprising the steps of:

a. selecting biological particles with functional receptors for Neisseria gonorrhoeae;

b. selecting a support;

c. depositing the selected biological particles on the selected support;

d. selecting an animal having at least one tissue;

e. placing the selected support on a tissue of the selected animal to juxtapose the selected biological particles deposited on the support with cells of the tissue of the selected animal;

f. placing biological particles deposited on the selected support in contact with cells of the tissue of the selected animal by means of a mechanical force applied to a surface of the support; and

g. subjecting the selected biological particles and cells of the tissue of the selected animal to an electric field.

66. The method of claim 65, further comprising the step of infecting the selected biological particles with Neisseria gonorrhoeae.

67. The method of claim 65 or 66, wherein the biological particles are selected from the group consisting of human HL60 or U937 lymphoma cells.

68. The method of claim 65 or 66, wherein the support is selected from the group consisting of filter paper and electrostatically-charged discs.

69. The method of claim 65 or 66, wherein the selected biological particles are deposited on the selected support by means of centrifugation.

70. The method of claim 65 or 66, wherein the selected animal is a rabbit.

71. The method of claim 65 or 66, wherein the tissue of the selected animal comprises corneal tissue.

72. The method of claim 65 or 66, wherein the tissue of the selected animal comprises histologically intact superficial corneal epithelial tissue.

73. The method of claim 65 or 66, wherein the mechanical force is applied with an electrode positioned on a surface of the support.

74. The method of claim 65 or 66, wherein the electric field comprises a direct current electric field.

75. The method of claim 74, wherein the direct current field is created by applying a pulse of direct current to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal.

76. The method of claim 74, wherein the direct current field is created by applying multiple pulses of direct current to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal.

77. A fusion product comprising biological particles fused to cells of animal or plant tissue produced in accordance with the method of any of claims 1-17.

78. A non-human animal characterized by features that differ from those typically displayed by the native species produced in accordance with the method of any of claims 18-34.

79. A plant characterized by features that differ from those typically displayed by the native species produced in accordance with the method of any of claims 18-34.

80. A non-human animal model for receptor-mediated pathogenic processes produced in accordance with the method of any of claims 35-53.

81. A plant model for receptor-mediated pathogenic processes produced in accordance with the method of any of claims 35-53.

82. A non-human animal model for gonorrhea produced in accordance with the method of any of claims 54-76.

83. A rabbit with selected biological particles fused to cells of its corneal tissue.

84. The rabbit of claim 83, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

85. The rabbit of claim 83, wherein the selected biological particles have functional receptors for Neisseria gonorrhoeae.

86. The rabbit of claim 83, wherein the selected biological particles are human HL60 cells with functional receptors for Neisseria gonorrhoeae.

87. The rabbit of claim 83, wherein the selected biological particles are fused to epithelial cells of its histologically intact superficial corneal epithelial tissue.

AMENDED CLAIMS

[received by the International Bureau on 27 September 1989 (27.09.89)
original claims 6,15,23,32,41,51,62 and 74 cancelled; claims 1,7-9,16-18,25,26,
33-35,42-44,52-54,63-65,75 and 76 amended other claims unchanged (15 pages)]

1. A method for fusing biological particles to cells of animal or plant tissue comprising the steps of:

a. selecting biological particles to be fused to cells of animal or plant tissue;

b. selecting animal or plant tissue;

c. placing the selected biological particles in contact with cells of the selected tissue; and

d. subjecting the selected biological particles and cells of the selected tissue to at least one direct current pulse.

2. The method of claim 1, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

3. The method of claim 1, wherein the selected animal or plant tissue comprises histologically intact animal or plant tissue.

4. The method of claim 1, wherein the selected biological particles are placed in contact with cells of the selected tissue by means of a mechanical force.

5. The method of claim 4, wherein the mechanical force is applied with an electrode juxtaposed with the selected biological particles and selected tissue.

6. The method of claim 1, wherein the electric field comprises a direct current electric field.

7. The method of claim 5, wherein the at least one direct current pulse is applied to an electrode juxtaposed with the selected biological particles and selected tissue.

8. The method of claim 5, wherein multiple pulses of direct current are applied to an electrode juxtaposed with the selected biological particles and selected tissue.

9. A method for fusing biological particles to cells of animal or plant tissue comprising the steps of:

a. selecting biological particles to be fused to cells of animal or plant tissue;

b. selecting a support;

c. depositing the selected biological particles on the selected support;

d. selecting animal or plant tissue;

e. placing the selected support on the selected tissue to juxtapose the selected biological particles deposited on the support with cells of the selected tissue;

f. placing the biological particles deposited on the selected support in contact with cells of the selected tissue by means of a mechanical force applied to a surface of the support; and

g. applying at least one direct current pulse to the selected biological particles and cells of the selected tissue.

10. The method of claim 9, wherein the biological particles are selected from the group consisting of animal

cells, plants cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

11. The method of claim 9, wherein the support is selected from the group consisting of filter paper and electrostatically-charged discs.

12. The method of claim 9, wherein the selected biological particles are deposited on the selected support by means of centrifugation.

13. The method of claim 9, wherein the selected animal or plant tissue comprises histologically intact animal or plant tissue.

14. The method of claim 9, wherein the mechanical force is applied with an electrode positioned on a surface of the support.

15. The method of claim 9, wherein the electric field comprises a direct current electric field.

16. The method of claim 15, wherein the at least one direct current pulse is applied to an electrode positioned on a surface of the selected support.

17. The method of claim 15, wherein multiple pulses of direct current are applied to an electrode positioned on a surface of the selected support.

18. A method for producing animals or plants characterized by features that differ from those typically displayed by the native species comprising the steps of:

a. selecting biological particles;

b. selecting an animal or plant having at least one tissue;

c. placing the selected biological particles in contact with cells of a tissue of the selected animal or plant; and

d. subjecting the selected biological particles and cells of the tissue of the selected animal or plant to at least one direct current pulse.

19. The method of claim 18, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

20. The method of claim 18, wherein the tissue of the selected animal or plant comprises histologically intact animal or plant tissue.

21. The method of claim 18, wherein the selected biological particles are placed in contact with cells of the tissue of the selected animal or plant by means of a mechanical force.

22. The method of claim 21, wherein the mechanical force is applied with an electrode juxtaposed with the selected

biological particles and cells of the tissue of the selected animal or plant.

24. The method of claim 23, wherein the at least one direct current pulse is applied to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal or plant.

25. The method of claim 23, wherein multiple pulses of direct current are applied to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal or plant.

26. A method for producing animals or plants characterized by features that differ from those typically displayed by the native species comprising the steps of:

a. selecting biological particles;

b. selecting a support;

c. depositing the selected biological particles on the selected support;

d. selecting an animal or plant having at least one tissue;

e. placing the selected support on a tissue of the selected animal or plant to juxtapose the selected biological particles deposited on the support with cells of the tissue of the selected animal or plant;

f. placing the biological particles deposited on the selected support in contact with cells of the tissue of the selected animal or plant by means of a mechanical force applied to a surface of the support; and

g. subjecting the selected biological particles and cells of the tissue of the selected animal or plant to at least one direct current pulse.

27. The method of claim 26, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

28. The method of claim 26, wherein the support is selected from the group consisting of filter paper and electrostatically-charged discs.

29. The method of claim 26, wherein the selected biological particles are deposited on the selected support by means of centrifugation.

30. The method of claim 26, wherein the tissue of the selected animal or plant comprises histologically intact animal or plant tissue.

31. The method of claim 26, wherein the mechanical force is applied with an electrode positioned on a surface of the support.

32. The method of claim 26, wherein the electric field comprises a direct current electric field.

33. The method of claim 32, wherein the at least one direct current pulse is applied to an electrode positioned on a surface of the selected support.

34. The method of claim 32, wherein multiple pulses of direct current are applied to an electrode positioned on a surface of the selected support.

35. A method for producing animal or plant models for receptor-mediated pathogenic processes comprising the steps of:

a. selecting biological particles with functional receptors for a pathogen;

b. selecting an animal or plant having at least one tissue;

c. placing the selected biological particles in contact with cells of a tissue of the selected animal or plant; and

d. subjecting the selected biological particles and cells of the tissue of the selected animal or plant to at least one direct current pulse.

36. The method of claim 35, further comprising the step of infecting the selected biological particles with the pathogen.

37. The method of claim 35 or 36, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes,

phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

38. The method of claim 35 or 36, wherein the tissue of the selected animal or plant comprises histologically intact animal or plant tissue.

39. The method of claim 35 or 36, wherein the selected biological particles are placed in contact with cells of the tissue of the selected animal or plant by means of a mechanical force.

40. The method of claim 39, wherein the mechanical force is applied with an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal or plant.

41. The method of claim 35 or 36, wherein the electric field comprises a direct current electric field.

42. The method of claim 41, wherein the at least one direct current pulse is applied to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal or plant.

43. The method of claim 41, wherein multiple pulses of direct current are applied to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal or plant.

44. A method for producing animal or plant models for receptor-mediated pathogenic processes comprising the steps of:

a. selecting biological particles with functional receptors for a pathogen;

b. selecting a support;

c. depositing the selected biological particles on the selected support;

d. selecting an animal or plant having at least one tissue;

e. placing the selected support on a tissue of the selected animal or plant to juxtapose the selected biological particles deposited on the support with cells of the tissue of the selected animal or plant;

f. placing the biological particles deposited on the selected support in contact with cells of the tissue of the selected animal or plant by means of a mechanical force applied to a surface of the support; and

g. subjecting the selected biological particles and cells of the tissue of the selected animal or plant to at least one direct current pulse.

45. The method of claim 44, further comprising the step of infecting the selected biological particles with the pathogen.

46. The method of claim 44 or 45, wherein the biological particles are selected from the group consisting of animal cells, plant cells, microorganisms, bacteria, yeast cells, liposomes, cell vesicles, cell organelles, lysosomes, phagosomes, nuclei, mitochondria, Golgi bodies, chloroplasts and vacuoles.

47. The method of claim 44 or 45, wherein the support is selected from the group consisting of filter paper and electrostatically-charged discs.

48. The method of claim 44 or 45, wherein the selected biological group particles are deposited on the selected support by means of centrifugation.

49. The method of claim 44 or 45, wherein the tissue of the selected animal or plant comprises histologically intact animal or plant tissue.

50. The method of claim 44 or 45, wherein the mechanical force is applied with an electrode positioned on a surface of the support.

51. The method of claim 44 or 45, wherein the electric field comprises a direct current electric field.

52. The method of claim 51, wherein the direct current pulse is applied to an electrode positioned on a surface of the selected support.

53. The method of claim 51, wherein multiple pulses of direct current are applied to an electrode positioned on a surface of the selected support.

54. A method for producing animal models for gonorrhea comprising the steps of:

a. selecting biological particles with functional receptors for Neisseria gonorrhoeae;

b. selecting an animal having at least one tissue;

c. placing the selected biological particles in contact with cells of a tissue of the selected animal; and

d. subjecting the selected biological particles and cells of the tissue of the selected animal to at least one direct current pulse.

55. The method of claim 54, further comprising the step of infecting the selected biological particles with Neisseria gonorrhoeae.

56. The method of claim 54 or 55, wherein the biological particles are selected from the group consisting of human HL60 or U937 lymphoma cells.

57. The method of claim 54 or 55, wherein the selected animal is a rabbit.

58. The method of claim 54 or 55, wherein the tissue of the selected animal comprises corneal tissue.

59. The method of claim 54 or 55, wherein the tissue of the selected animal comprises histologically intact superficial corneal epithelial tissue.

60. The method of claim 54 or 55, wherein the selected biological particles are placed in contact with cells of the tissue of the selected animal by means of a mechanical force.

61. The method of claim 60, wherein the mechanical force is applied with an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal.

62. The method of claim 54 or 55, wherein the electric field comprises a direct current electric field.

63. The method of claim 62, wherein the at least one direct current pulse is applied to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal.

64. The method of claim 62, wherein multiple pulses of direct current are applied to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal.

65. A method for producing animal models for gonorrhea comprising the steps of:

a. selecting biological particles with functional receptors for Neisseria gonorrhoeae;

b. selecting a support;

c. depositing the selected biological particles on the selected support;

d. selecting an animal having at least one tissue;

e. placing the selected support on a tissue of the selected animal to juxtapose the selected biological particles deposited on the support with cells of the tissue of the selected animal;

f. placing biological particles deposited on the selected support in contact with cells of the tissue of the selected animal by means of a mechanical force applied to a surface of the support; and

g. subjecting the selected biological particles and cells of the tissue of the selected animal to at least one direct current pulse.

66. The method of claim 65, further comprising the step of infecting the selected biological particles with Neisseria gonorrhoeae.

67. The method of claim 65 or 66, wherein the biological particles are selected from the group consisting of human HL60 or U937 lymphoma cells.

68. The method of claim 65 or 66, wherein the support is selected from the group consisting of filter paper and electrostatically-charged discs.

69. The method of claim 65 or 66, wherein the selected biological particles are deposited on the selected support by means of centrifugation.

70. The method of claim 65 or 66, wherein the selected animal is a rabbit.

71. The method of claim 65 or 66, wherein the tissue of the selected animal comprises corneal tissue.

72. The method of claim 65 or 66, wherein the tissue of the selected animal comprises histologically intact superficial corneal epithelial tissue.

73. The method of claim 65 or 66, wherein the mechanical force is applied with an electrode positioned on a surface of the support.

74. The method of claim 65 or 66, wherein the electric field comprises a direct current electric field.

75. The method of claim 74, wherein the at least one direct current pulse is applied to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal.

76. The method of claim 74, wherein multiple pulses of direct current are applied to an electrode juxtaposed with the selected biological particles and cells of the tissue of the selected animal.

77. A fusion product comprising biological particles fused to cells of animal or plant tissue produced in accordance with the method of any of claims 1-17.

78. A non-human animal characterized by features that differ from those typically displayed by the native species produced in accordance with the method of any of claims 18-34.

79. A plant characterized by features that differ from those typically displayed by the native species produced in accordance with the method of any of claims 18-34.

80. A non-human animal model for receptor-mediated pathogenic processes produced in accordance with the method of any of claims 35-53.

81. A plant model for receptor-mediated pathogenic processes produced in accordance with the method of any of claims 35-53.

82. A non-human animal model for gonorrhea produced in accordance with the method of any of claims 54-76.

Ganser, Bischoff and Podesta all disclose the use of alternating current which results in the phenomenon of dielectrophoresis. See the first page (page 225) of Ganser, the second page (page 65), first column, of Bischoff, and the first page (page 329), second column, of Podesta.

In sharp contrast, Applicant's claimed method does not use an alternating current field at all and does not harness the effects of the phenomenon of dielectrophoresis or rely on said phenomenon in any way. Applicant must state his invention in positive terms, so he cannot amend his claim by adding "in the absence of alternating current and in the absence of dielectrophoresis," of course. Instead, the independent claims of the application have been amended to positively recite with increased particularity what applicant's invention is. As now claimed, neither Ganser, Bischoff or Podesta teach or suggest the invention.

The references to direct current pulses in Ganser, Bischoff or Podesta neither teach nor suggest Applicant's invention because said disclosures teach the use of direct current pulses after the dielectrophoresis phenomenon has occurred in response to the application of the alternating current field. As pointed out in Applicant's discussion of the prior art, the alternating current is destructive to biological particles. Applicant's use of direct current pulses and mechanical pressure completely eliminates the need for alternating current, provides a method of electrofusing that does not rely on the phenomenon of dielectrophoresis, and as such represents a major technological breakthrough.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

I.P.C. (4) A01K 45/00
US. Cl. 800/1

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	604/20 128/362,419R,897 800/1

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

BIOLOGICAL ABSTRACTS, CHEMICAL ABSTRACTS, MED-LINE

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Marme et al, Plasmalemma and Tonopast: Their Functions in the Plant Cell, 1982, Elsevier Biomedical Press, article by Gasner et al pages 225-231.	1,2,5-10, 15-19,22-27,31-35, 37,41-44, 46,50-54, 62-64,79 and 81 4,11,12, 14,21,28, 29,40,47, 48,56-58 60,61,65 and 67-76

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 July 1989	26 JUL 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	John E. Tarcza

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X Y	FEBS Letters, 147(1) October 1982, Bischoff et al, "Human Hybridoma Cells Produced by Electrofusion" Pages 64-68	1,2,5-10, 15-19,22-27,31-35, 37,41-44, 46,50-54, 62-64,77, 78,80 and 82 4,11,12, 14,21,28, 29,39,40, 47,48,56-58,60,61, 65 and 67-76

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Y	BIOCHEMI[illegible]L AND BIOSPHYSICAL RESEA[illegible] COMMUNICATIONS, 140(1) 15 October 1986 Teissie et al "Fusion of Mammalian cells in Culture in Obtained by Creating the Contact Between Cells after their Electropermeabilization" pages 258-266.	4,12,14, 21,29,39, 40,48,60, 61,65,67 and 69-76
X Y	EUROPEAN JOURNAL OF BIOCHEMISTRY 145, 1984, Podesta et al, "Production of Steroid Hormone and Cyclic AMP in Hybrids of Adrenal and Leydig Cells Generated by Electrofusion," pages 329-332.	1,2,5-10, 15-19,22-27,31-35, 41-44,46, 50-54,62-64,77,78, 80 and 82
		4,11,12, 14,21,28, 29,39,40, 47,48,56-58,60,61, 65 and 67-76
X Y	JOURNAL OF IMMUNOLOGY, 132(1), January 1984, Correa-Freire et al, "Introduction of HLA-A/B Antigens into Lymphoid Cell Membranes by Cell-Liposome Fusion" pages 69-75.	77,78,80 and 82
		1,2,4-12 14-19,21 -29,31-35,37,39-44,46-48,50-54,56-58, 60-65, and 67-76
Y,P	US,A, 4,786,277 (POWERS) 22 November 1988. See column 31 line 64-69 in particular.	11,28, 47 and 68.
Y	US,A, 4416,274(JACOBSEN) 22 November 1983. See the abstract.	11,28 47 and 68

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